

Award Number:
W81XWH-08-1-0385

TITLE:
Metabolic stress induced by arginine deprivation induces autophagy cell
death in prostate cancer

PRINCIPAL INVESTIGATOR:
Richard Bold, MD

CONTRACTING ORGANIZATION:
University of California, Davis
Davis, CA 95618

REPORT DATE:
August 2010

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 31/08/2010		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 Aug 2009 - 31 Jul 2010	
4. TITLE AND SUBTITLE Metabolic stress induced by arginine deprivation induces autophagy cell death in prostate cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0385	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Richard Bold Email: richard.bold@ucdmc.ucdavis.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California Davis Office of Research 1850 Research Park Drive, Suite 300 Davis, CA 95618				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The primary purpose of this research grant is to provide the necessary preclinical data demonstrating that prostate cancer cells are auxotrophic for arginine and therefore targeting arginine metabolism is a novel therapeutic approach. The primary methodology involves cell culture with the characterization of the arginine requirements for prostate cancer cell growth and then determination of the effect of arginine depletion on cell growth and cell death. Furthermore, we have investigated the mechanism of cell death and observed that arginine deprivation in those cells auxotrophic for this semi-essential amino acid induces autophagy as a precursor to programmed cell death. Major findings to date include the observation that the majority of prostate cancer cell lines lack arginine-succinate synthetase (ASS), the critical enzyme in arginine biosynthesis. Furthermore, arginine deprivation in those cell lines lacking ASS induces autophagy as a precursor to non-apoptotic cell death. Inhibition of autophagy appears to stimulate the induction of cell death.					
15. SUBJECT TERMS Prostate cancer, autophagy, arginine deiminase					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction

This is the second year of a three year grant entitled “Metabolic stress induced by arginine deprivation induces autophagy cell death in prostate cancer”. The **primary hypothesis** of the research investigation is PEG-ADI represents a potential therapy of prostate cancer to induce metabolic stress by arginine deprivation and subsequent induction of autophagic cell death as a precursor to apoptosis in those prostate cancers lacking biosynthetic enzymes to make arginine. The **specific aims** to investigate this hypothesis are:

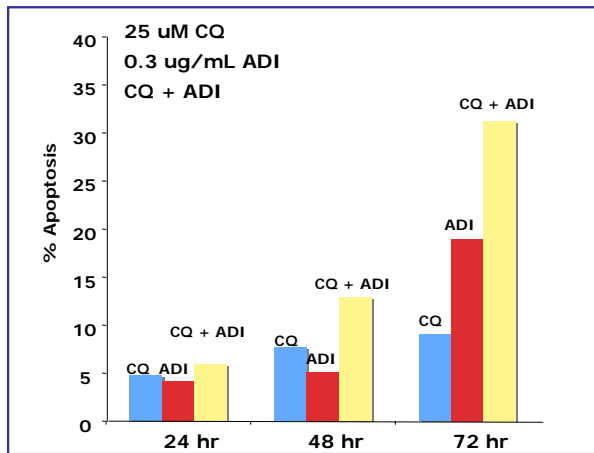
- 1) We will demonstrate that in those prostate cancer cells lacking ASS for which arginine is an essential amino acid, metabolic stress induced by arginine deprivation achieved by ADI-PEG treatment induces autophagic cell death as a precursor to apoptosis via a DRAM-dependent pathway. From these studies, we will show: 1) ASS expression predicts cellular response to PEG-ADI, 2) PEG-ADI therapy induces arginine deprivation, 3) the consequence of the metabolic stress is the initiation of autophagosome assembly and progression to autophagic cell death, and 4) DRAM induction is required for PEG-ADI induced autophagy.
- 2) We will demonstrate that the induction of PEG-ADI induced autophagy in prostate cancer cells is a precursor to apoptosis; furthermore, these events sensitize cells to traditional chemotherapy-induced apoptosis. Autophagy mediators will be altered to determine effect on sensitivity to PEG-ADI induced cellular events. In addition, PEG-ADI will be combined with traditional chemotherapy to determine effect on apoptosis and in vivo tumor response.

Body

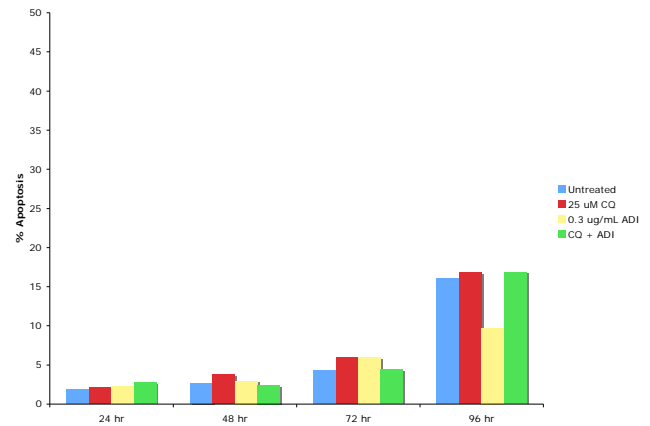
Significant progress has been accomplished during the second year of this 3-year award. The initial focus of the investigation was the characterization of the auxotrophic requirements for prostate cancer cell lines for the semi-essential amino acid arginine. These studies clearly demonstrated that only those prostate cancer cell lines lacking argininosuccinate synthetase (ASS) were sensitive to the cytotoxic effect of PEG-ADI (1). Furthermore, PEG-ADI induced autophagy as well as a caspase-independent cell death (2), though the relationship between these two cellular consequences of arginine are unclear. Therefore, the second year of the grant focused on the role of autophagy in the cell death as well as the intracellular signaling mechanisms responsible for both the induction of autophagy and cell death.

Chloroquine is a well recognized inhibitor of autophagy by blocking the final fusion of the autophagosome to the lysosome and is a common biochemical method used to inhibit autophagy. Therefore, to determine the role of autophagy in the cell death mediated by ADI, the ASS-deficient CWR22RV1 and the ASS-expressing LNCaP prostate cancer cells were treated with PEG-ADI in the absence or presence of chloroquine (ChQ). As noted previously, PEG-ADI induces cell death only in the ASS-deficient CWR22RV1 cell line but without effect on LNCaP cells (Figure, below). However, chloroquine both accelerated the cell death and increased the cell death following PEG-ADI treatment in CWR22RV1 cells (Figure, below), without effect on LNCaP cells. These data indicate that the autophagy initiated by PEG-ADI plays a cell survival role, inhibiting the delayed cell death. Therefore, the anti-cancer efficacy of PEG-ADI in prostate cancer may ultimately be increased by inhibitors of autophagy, and as chloroquine is a safe, accepted therapy in the treatment of malaria, these findings have significant impact on the further clinical development of PEG-ADI in prostate cancer.

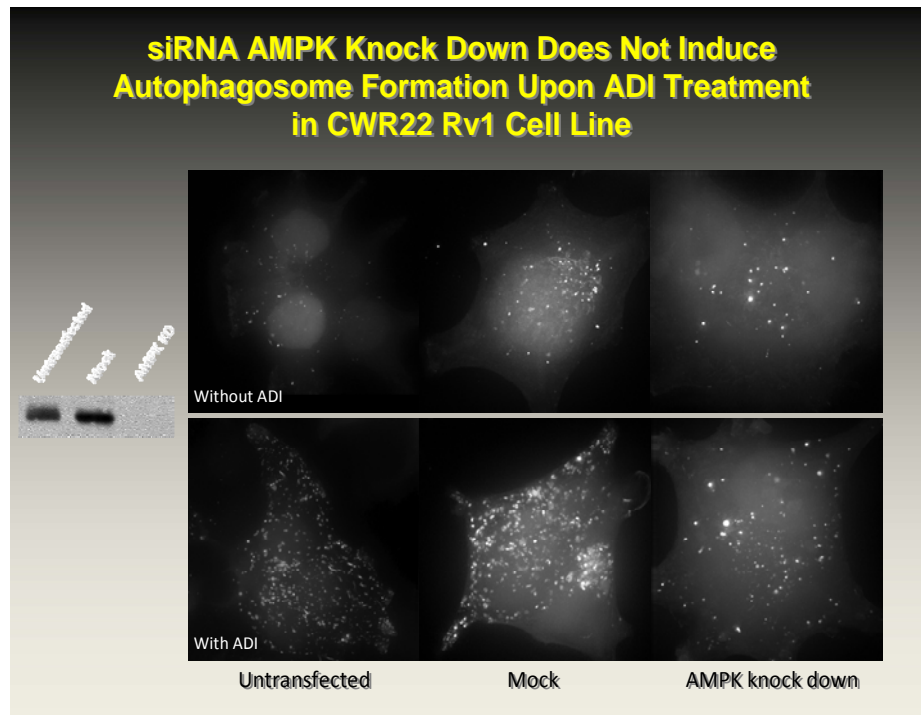
Chloroquine increases effect of ADI on CWR22Rv1



Chloroquine and ADI have no effect on LNCaP

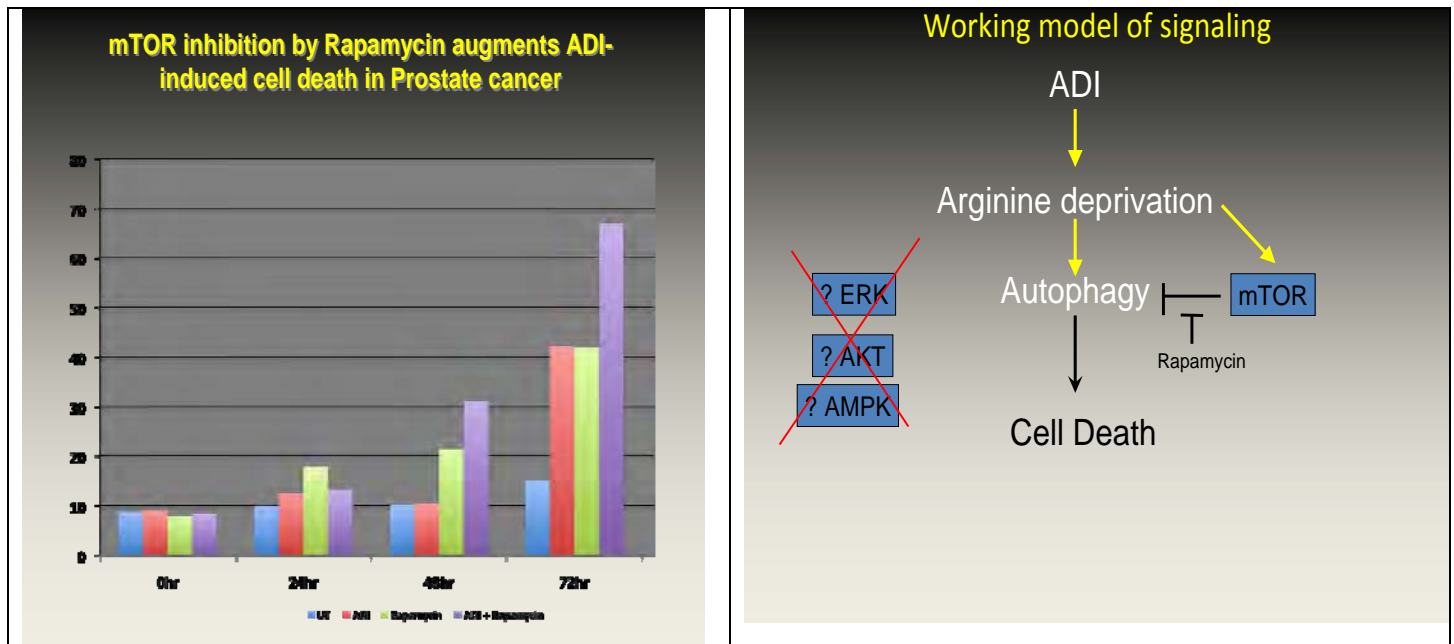


The second direction of our research is the investigation of the signaling mechanisms that mediate the cellular events of autophagy and cell death following PEG-ADI in prostate cancer. We began by inhibition of pathways that have either been demonstrated to be important in the initiation of autophagy (e.g. AMP kinase, AKT, rag), or were observed to be activated following PEG-ADI treatment (e.g. ERK1/2, mTOR). Using either small molecule inhibitors, or siRNA-mediated knockdown, these five signaling intermediates were inhibited in to determine the effect on both induction of autophagy and cell death. Interestingly, none of the autophagy pathways (e.g. AMP kinase, AKT, rag) regulated cell death, as inhibition of each did not affect the induction of cell death mediated by PEG-ADI in CWR22RV1 cells (data not shown). Those these pathways did inhibit the induction of autophagy, as measures by immunofluorescence for LC-3, a protein involved in the assembly of the autophagosome (Figure, right). In the presence of PEG-ADI (lower panel), the diffuse cytoplasmic staining of LC-3 became more punctate, which is representative of autophagosome formation. When AMP kinase was knocked down by siRNA (immunoblot shown to demonstrate absence of protein), far fewer punctae were observed, consistent with an inhibition of autophagy (lower right panel). As a side note, the methodology that we have developed for the examination of autophagy is that of live single cell, continuous immunofluorescence. This technology is currently the subject of a manuscript in preparation (3).

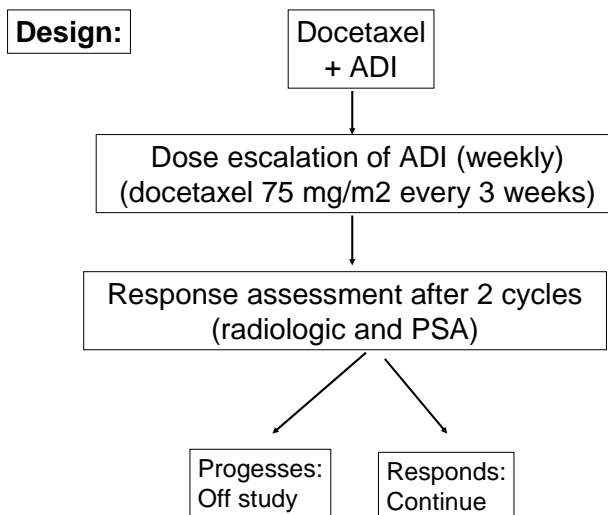


When we examined the mTOR pathway, which is activated upon treatment with PEG-ADI, we noted two key observations. First, unlike inhibition of the other pathways examined, inhibition of mTOR with rapamycin initiated autophagy. Secondly, rapamycin treatment was sufficient to induce cell death. Therefore, the cellular effects of rapamycin appeared to be similar to PEG-ADI, namely induction of autophagy and cell death. When these two treatments occurred simultaneously in CWR22RV1, the subsequent cell death was increased, with nearly additive effects (Figure, below left panel). These

findings have led us to develop a hypothesis that while mTOR is activated by PEG-ADI, it is neither sufficient nor required for the cell death mediated by PEG-ADI (Figure below, right panel).



Lastly, given our very convincing data combining PEG-ADI with docetaxel in a murine xenograft model of prostate cancer, we have completed preparation of a human subjects protocol to conduct a Phase I study of this combination in hormone-refractory prostate cancer. The protocol is currently undergoing IRB review at UCDavis and has the full support of Polaris Pharmaceuticals, the manufacturer of PEG-ADI (see Appendix). In addition, we plan to submit a R21 grant application to the NIH to support this clinical trial (submission of November 2010, Dr. Primo Lara, Principal Investigator).



Key Research Accomplishments

1. Characterization of signaling pathways mediating autophagy and cell death following PEG-ADI in prostate cancer.
2. Development of real-time single cell imaging for assessment of autophagy.
3. Investigation of the interaction of autophagy and cell death mediated by PEG-ADI

Reportable Outcomes

Manuscripts:

1. Changou, A. G.P. McNerney, J.U. Jung, F.Y.S. Chuang, H-J. Kung **R.J. Bold**. Real time immunofluorescence for imaging and quantification of autophagy. Manuscript in preparation.
2. Changou A., Kim, R.H., J.M. Coates, J.U. Jung, F.Y.S. Chuang, **R.J. Bold**, and H-J. Kung. Signaling pathways mediating autophagy induced by metabolic stress in human prostate cancer. Manuscript in preparation.

Grant submission:

NIH R21 (pending designation, November 2010 submission). A Phase I clinical trial of docetaxel and PEG-ADI in hormone refractory prostate cancer.

Conclusion

In our second of a three year grant entitled “Metabolic stress induced by arginine deprivation induces autophagy cell death in prostate cancer”, we have made significant progress in the investigation of our central hypothesis. This has allowed us to move forward to a clinical trial of PEG-ADI in prostate cancer, which is a very noteworthy goal within 2 years of preclinical drug development. Furthermore, we have begun to elucidate signaling pathways involved in the cell death of PEG-ADI, which can be important information for additional drug development, as two of the inhibitors (chloroquine and rapamycin) are already in human use.

As far as the financial conduct of the research, we are well within the budget without any significant deviations noted or anticipated.

References

3. Kim, R.H., J.M. Coates, T.L. Bowles, G.P. McNerney, J. Sutcliffe, J.U. Jung, R. Gandour-Edwards, F.Y.S. Chuang, **R.J. Bold**, and H-J. Kung. Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. *Cancer Research*, 69(2):700-708, 2009.
4. Kim, R.H., **R.J. Bold**, and H-J. Kung. ADI, autophagy and apoptosis: Metabolic stress as a therapeutic option for prostate cancer. *Autophagy*. 5(4):567-8, 2009.
5. Changou, A. G.P. McNerney, J.U. Jung, F.Y.S. Chuang, H-J. Kung **R.J. Bold**. Real time immunofluorescence for imaging and quantification of autophagy. Manuscript in preparation.



Polaris Group

6370 Nancy Ridge Drive, Suite 106
San Diego, CA 92121 U.S.A.
858.452.6688
Fax: 858.452.3193

5 July 2010

Primo N. Lara, Jr., MD
Professor of Medicine
Associate Director of Translational Research
University of California Davis Cancer Center
4501 X Street
Sacramento, CA 95817

Re: Grant Application "Phase I trial of ADI-PEG-20 plus docetaxel in advanced solid tumors with emphasis on castration resistant prostate cancer (CRPC)"

Dear Dr. Lara:

This is to confirm that we are willing to provide the study drug, ADI-PEG 20, for the above noted protocol without charge. We are very pleased by the prospect of exploring the potential utility of arginine depletion with our pegylated arginine deiminase (ADI-PEG 20) in combination therapy with docetaxel, especially in subjects with castration resistant prostate cancer. Exciting pre-clinical work from your institution has demonstrated that ADI-PEG 20, especially in combination with docetaxel, may be of benefit in prostate cancer.

Also please note that we have our own manufacturing facility, DesignRx Pharmaceuticals, Inc., which has been producing ADI-PEG 20 for our current phase 2 clinical studies, and will also produce ADI-PEG 20 for our planned global, phase 3 study in hepatocellular carcinoma. In addition, we have sufficient financial resources to produce ADI-PEG 20 for your study.

If any further clarification is needed, please do not hesitate to contact me.

With best regards,

John S. Bomalaski, M.D.
Executive Vice-President, Medical Affairs